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Communication

Abscisic Acid Accumulation Is Not Required for Proline Accumulation in Wilted Leaves[1]

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ABSTRACT

Leaves from dark-grown barley (*Hordeum vulgare* L. var Larker) seedlings grown in the presence and absence of fluridone were used to determine whether or not abscisic acid (ABA) accumulation was necessary for proline to accumulate in wilted tissue. Wilted tissue (polyethylene glycol-treated) leaves from fluridone-grown seedlings did not accumulate ABA but did accumulate proline at a rate that was not different from the non-fluridone-treated leaves. Thus ABA accumulation is not required for wilting-induced proline accumulation in barley leaves. Proline accumulation in wilted leaves from the wilty tomato (*Lycopersicon esculentum*) mutant, *flacca*, was compared to that in the wild type, Rheinlands Ruhm. Proline accumulated in wilted leaves from *flacca*. The rate of accumulation was faster in *flacca* compared to the rate in the wild type because the wilty mutant wilted faster. ABA accumulated in wilted leaves from the wild type but not in the wilty mutant. This result is a further confirmation that ABA accumulation is not required for wilting-induced proline accumulation. These results are significant in that proline accumulation in barley leaves can be induced independently by any one of three treatments: wilting, ABA, or salt.

Both ABA and proline accumulations are widely recognized metabolic responses to stress in plants (8). ABA accumulates in response in turgor loss (5) and proline accumulates under drought, salt, and cold stresses (2). Proline also accumulates in response to exogenous ABA treatment, but only in certain species (1, 13).

The goal of our research is to understand the metabolic and cellular processes involved in proline accumulation. We have shown that proline accumulation involves increased synthesis and slower utilization of proline by oxidation and protein synthesis (4, 13, 14). In wilted barley leaves, ABA accumulation precedes proline accumulation. Also in wilted barley leaves, proline has not previously been observed to accumulate in the absence of ABA accumulation (15, 16). In salt-shocked barley leaves, proline does accumulate in the absence of ABA accumulation (15).

Both ABA and proline accumulation are prevented by inhibitors of transcription and translation (7, 16). This result suggests that gene activation is required for these compounds to accumulate. Benzyladenine prevents wilt-, ABA-, and salt-induced proline accumulation but has no effect on ABA accumulation in wilted leaves (16). Thus some cellular processes are common to proline accumulation in all these treatments. However, since salt induces proline accumulation in the absence of ABA accumulation, there also may be differences in the cellular processes involved under these treatments.

We have attempted to answer the question of whether or not ABA accumulation is required for proline accumulation in wilted leaves by chemically inhibiting ABA accumulation in barley. We have tried benzyladenine (10, 16) and compounds that inhibit ABA synthesis in fungi such as ancymidol (12) and paclobutrazol (11), even though ABA biosynthesis in higher plants occurs by a different pathway. None of these has inhibited ABA accumulation in wilted barley leaves.

It has recently been reported that the herbicide, fluridone, inhibits wilting-induced ABA accumulation in etiolated barley seedlings grown in such a way that pools of carotenoid precursors are very low (6). Norflurazon, another inhibitor of carotenoid biosynthesis, also inhibits wilt-induced ABA accumulation in *Pennisetum americanum* shoots (9). Fluridone, a pyridinone, and norflurazon, a pyridazinone, are chemically similar herbicides.

A second approach to the question of the role of ABA in wilting-induced proline accumulation is to make use of ABA-less or ABA-deficient mutants. Several wilty mutants of tomato are available as well as some in other species. Perhaps the best known and characterized wilty mutant is the tomato, *flacca*. This mutant contains very low levels of ABA, does not accumulate it in response to stress and responds to stress like the wild type when ABA is applied. When grown under high humidity or in the presence of added ABA, it is essentially indistinguishable from the wild type.

In this paper we report proline accumulation in wilted, etiolated barley leaves treated with fluridone. Further we report proline accumulation in wilted leaves of the wilty tomato mutant, *flacca*. Thus it appears that ABA accumulation is not required for wilting induced proline accumulation.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. var Larker) seeds were imbibed and germinated as described by Gamble and Mullett (6) in the presence and absence of 0.1 mM fluridone (Eli Lilly Research Laboratories). Tween (0.5%) was used to solubilize the fluridone to be added to the vermiculite as well as that in which the seeds were soaked.

Seeds of *Lycopersicon esculentum* Mill. var Rheinlands Ruhm (wild type) and *flacca* (wilty) were obtained from Dr. Charles Rick, University of California, Davis. These tomato seeds were soaked in 2.5% (w/v) sodium hypochlorite, then germinated in soil. Then small plants were transplanted into 25 cm i.d. pots

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and grown in the greenhouse during the summer under a mist system. Seeds were harvested from ripe fruits and air dried. Healthy plants of *flacca* were essentially morphologically identical to the wild type when both were grown this way. Experiments were conducted with excised mature leaves.

First leaves of 8- to 10-day-old etiolated barley seedlings grown in the presence and absence of fluridone were excised and cut into 1 cm segments. All leaves were pretreated by floating them on 50 mM sucrose and 1 mM L-glutamate. Leaves were wilted by incubation on 350 g/L PEG containing sucrose and glutamate. These wilted leaves lost about 25% of their original weight during the experiment. Control leaves were incubated on sucrose and glutamate only and they maintained turgor as indicated by maintaining their original weight. Incubations were in darkness.

Mature leaves of tomato were excised from plants at the end of the light period and allowed to take up 50 mM sucrose and 1 mM L-glutamate through the cut petiole for 12 h in room light. Leaves were wilted by allowing the leaflets to dry to 75% of their original fresh weight on the laboratory bench under a 100 W incandescent lamp placed about 30 cm above the leaves. Control leaves were maintained at their original fresh weight by keeping them in a humid box.

Extraction, purification, and quantitative determination of proline (14) and ABA (16) was previously described for barley leaves. The same procedure was used for ABA determination of tomato leaves but proline was determined by the method of Bates *et al.* (3).

RESULTS

The levels of free proline in barley leaves grown in the presence and absence of fluridone and incubated in a wilted or turgid condition are shown in Figure 1B. Leaves from fluridone-treated plants were white whereas leaves from untreated seedlings were yellow, typical of etiolated barley leaves. The results in Figure 1B show that wilted etiolated leaves accumulate proline and that growth of the seedlings in the presence of fluridone has no effect on wilting-induced proline accumulation.

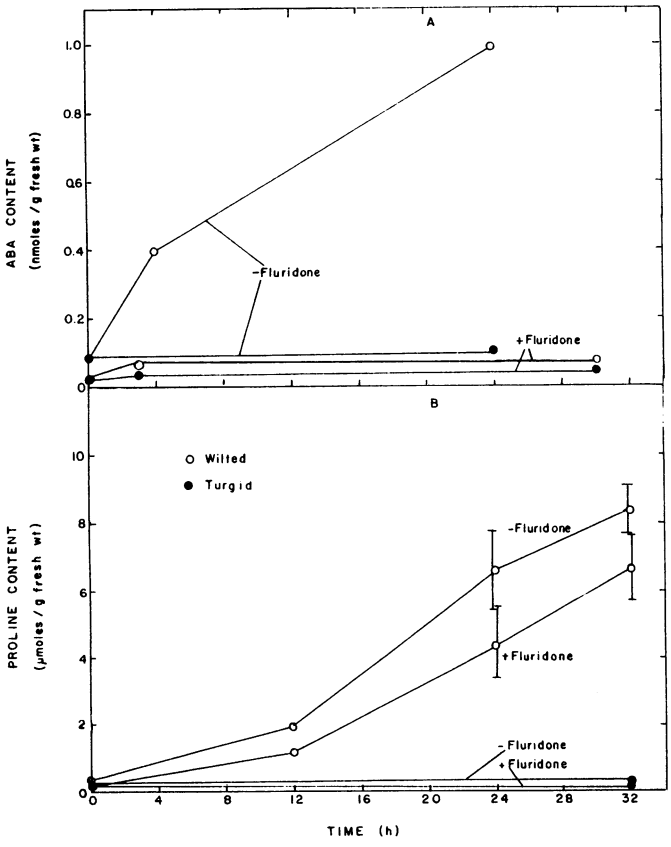

FIG. 1. Levels of ABA (A) and proline (B) in barley leaves dark-grown in the presence and absence of fluridone when incubated in darkness for varying time periods on 50 mM sucrose and 1 mM glutamate (turgid) or the same solution containing 350 g/L PEG (wilted). Error bars represent standard deviations (three replicates).

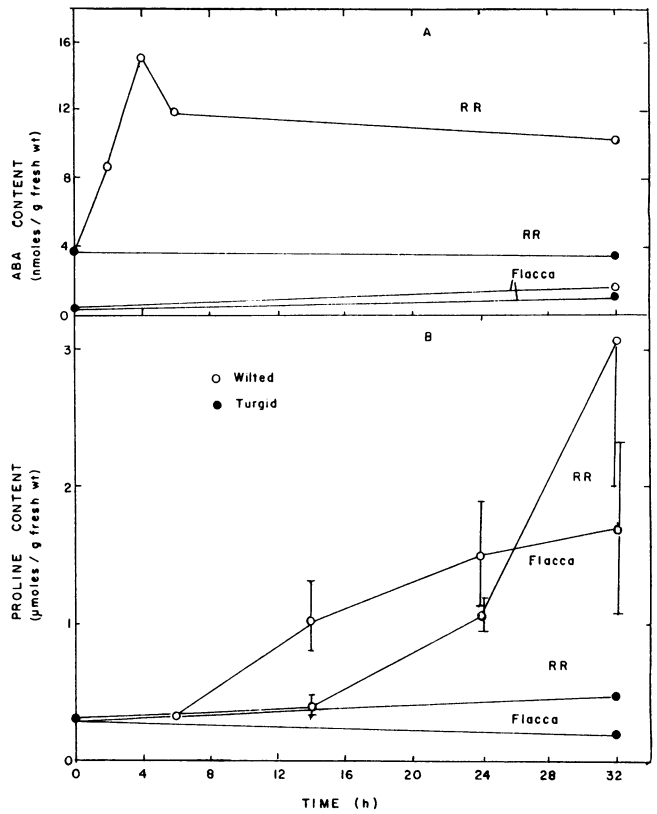

FIG. 2. Levels of ABA (A) and proline (B) in leaves from the wilty tomato mutant, *flacca*, and the wild type, Rheinlands Ruhm (RR), incubated for varying time periods in a wilted and turgid condition. Error bars represent standard deviations (three replicates).

Detectable levels of ABA were found in leaves from seedlings grown on fluridone (Fig. 1A). However, these levels are clearly below the level required to cause proline to accumulate in barley (15) and below the level found in wilted barley leaves in which proline accumulated (15). The data in Figure 1 clearly show proline accumulation in barley tissue in which the ABA level is at or below the level normally found in turgid tissue (6, 15).

Proline levels in leaves from the wilty tomato, *flacca*, and the wild type, Rheinlands Ruhm, incubated in wilted and turgid conditions are shown in Figure 2B. These levels indicate that wilting induces proline to accumulate in these leaves and that the wilty mutant accumulates proline at least as fast as the wild type. The higher level of proline in *flacca* compared to the wild type at the 14 h sampling time is due to a more rapid wilting of the *flacca* leaves. The ABA levels from similarly treated leaves shown in Figure 2A indicate that the wild-type wilted leaves accumulate ABA but that those from *flacca* do not.

DISCUSSION

The results presented in this paper show that proline accumulates in wilted leaves in the absence of previous ABA accumulation, and therefore clearly indicate that ABA and proline accumulations are not causally linked. The time course and dose response correlations which we previously reported (15) are just that: correlations that do not result from any link between the two accumulations. Proline can accumulate in wilted leaves that

do not contain elevated levels of ABA or salt, it can accumulate in turgid leaves in response to ABA but in the absence of added salt, and it can accumulate in turgid leaves in response to salt but in the absence of ABA accumulation (15). We can conclude that each of these three treatments all have some component that is independent of the other two. However, there are probably metabolic or cellular processes that are common to all three treatments because proline accumulation under all of them is inhibited by cycloheximide, cordycepin, and benzyladenine (16). Furthermore, proline accumulation in all cases involves increased proline synthesis from precursors (4, 13, 14).

That proline accumulates in the presence of fluridone, which inhibits ABA accumulation, is further evidence that fluridone-treated leaves have metabolic capabilities beyond that required for growth in the dark. These results support the claim by Gamble and Mullett (6) that fluridone inhibition of ABA accumulation is not due to some secondary effect such as plastid dysfunction that would result in the inability to produce ATP and reducing power. That proline accumulation is sensitive to inhibitors of transcription and translation but not to fluridone indicates that fluridone treated leaves can synthesize RNA and protein *de novo* in response to stress in addition to macromolecular synthesis required for growth.

These results also contribute to our understanding of the mechanism of the previously reported inhibition of wilting-induced proline accumulation by cordycepin and cycloheximide. Since ABA accumulation is also inhibited by cordycepin and cycloheximide (6, 15), then inhibition of proline accumulation by these inhibitors could be due to the inhibition of ABA accumulation if ABA accumulation were an obligatory step leading to proline accumulation. Since, however, the results reported here indicate that proline accumulates even when there is no ABA accumulation, cordycepin and cycloheximide must directly affect proline accumulation. This effect is interpreted as further evidence that wilting has a specific effect on the expression of genes regulating proline metabolism.

The lack of a link between ABA and proline accumulation is consistent with the observations previously reported that several species do not accumulate proline in response to exogenous ABA (1, 15). It is known that there is species variability in the rate at which proline accumulates (2). It would be interesting to know if there is a correlation between the rate at which proline accumulates and whether or not it accumulates in response to added ABA.

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